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(54) Title: ANTIBODIES TO STREPTOLYSIN O DERIVATIVES AND VARIANTS (57) Abstract Disclosed are antibodies to derivatives and variants of Streptolysin O ("SLO"). Methodologies for generating such antibodies are further disclosed, as are purification protocols for wild-type SLO and assaying techniques for wild-type SLO.		

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ANTIBODIES TO STREPTOLYSIN O
DERIVATIVES AND VARIANTS

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RELATED APPLICATIONS

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The application is related to United States
Serial No. _____ (Beckman Docket No. 128D-104)
entitled "Streptolysin O Derivatives" by Craig W. Adams
and Eva Y. Wang and United States Serial No. _____
15 (Beckman Docket No. 128D-122) entitled "Streptolysin O
Variants", by Craig W. Adams. Both applications are
filed simultaneously herewith, and both are incorporated
herein by reference.

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FIELD OF THE INVENTION

The present invention is generally related to
Streptolysin O and particularly to Streptolysin O
antibodies of the monoclonal, polyclonal and recombinant-
25 DNA derived types. In its most particular aspect, the
present invention relates to antibodies to Streptolysin O
derivatives and variants produced by recombinant DNA
technology.

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BACKGROUND OF THE INVENTION

I. Streptolysin O

Streptolysin O ("SLO") has an approximate
35 molecular weight of between about 65,000 and about 70,000
daltons. SLO belongs to a class of oxygen sensitive

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("thiol-activated"), cell destroying ("cytolytic") toxin ("cytotoxin") which are produced by gram-positive bacterial species belonging to four different genera (streptococcus, bacillus, clostridium and listeria).

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SLO interacts with membrane cholesterol and exerts cytolytic-cytotoxic effects on a broad range of mammalian cells. Additionally, SLO has very potent cardiotoxic properties. One of the toxic and pathogenic properties associated with SLO is its hemolytic activity, i.e. SLO will lyse red blood cells, resulting in the release of hemoglobin. SLO can be lethal to laboratory animals in relatively small doses. Injection of SLO into an animal typically results in its immediate death. Most typically, SLO is associated with rheumatic fever in humans, an acute infectious disease characterized by fever, profuse perspiration, painful inflammation and swelling of the joints, and often inflammation of the lining membrane of the heart ("endocarditis").

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Because SLO is produced by specified bacterial species, when these species "invade" a mammalian host, the SLO released by the bacteria is treated by the host as a foreign protein. SLO, then, is an antigen. "Antigens" are high molecular weight compounds which upon entry into the blood stream of a vertebrate stimulate the transformation of the small lymphocytes of the B-type into lymphoblasts. The lymphoblasts secrete antibodies specific to the antigen stimulator. The antibodies are proteins possessing reactive sites specifically complementary to a reactive feature or site on the stimulating antigen. Antibodies generally have the property of rendering the antigen harmless to the host organism by occupying the immunologically active sites, or "epitopes", on the antigen particles or molecules. Anti-SLO antibodies ("ASO") are therefore produced by the

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determination of infection by SLO secreting bacteria involve nephelometric and turbidimetric protocols.

5 In order to utilize these immunodiagnostic
assaying techniques, it is necessary to have access to
sufficient SLO to be added to the mixture. One source of
SLO is the bacteria *Streptococcus pyogenes* ("S.
pyogenes"), whereby the SLO is secreted from S. pyogenes
into the culture broths. Experience has demonstrated
10 that the purification of SLO from its natural host is
difficult and expensive.

II. Antibodies: Monoclonal, Polyclonal
 and Recombinant DNA Technology Based

15 Monoclonal antibodies have a single antibody
specificity and affinity, as well as a single
immunoglobulin isotype (a protein fraction containing
antibody activity is referred to as an "immunoglobulin").
20 Polyclonal antibodies, on the other hand, contain a
variety of antibody molecules directed against an antigen
of interest, as well as antibodies which do not react
with the antigen of interest. Because of the specificity
and affinity associated with monoclonal antibodies, these
25 are generally preferred over polyclonal antibodies when
such specificity is needed, e.g., in purification
protocols. The specificity of monoclonal antibodies
allows one to identify and screen for monoclonal
antibodies which comprise the specific binding
30 characteristics of interest. For example, two monoclonal
antibodies can be selected to bind to the same antigen,
but at different locations. Such antibodies can be of
significant value in, e.g., sandwich-based immunoassays.

Briefly, the production of monoclonal antibodies comprises the following steps: Immunization; Hybridization; Propagation; Screening; and Cloning.

5 The immune response of an animal, e.g. a
mammal such as a mouse, injected with an antigen which it
has never encountered before will elicit a "Primary
Response", i.e., the animal's immune system will generate
a small amount of antibody against the antigen. If after
10 a time period the animal is reinjected with the antigen,
the immune system will elicit a "Secondary Response",
i.e., the response is faster, stronger (more antibody is
made), and qualitatively different from the Primary
Response (antibody which binds with a higher affinity to
15 the antigen will be generated). Generally, it is
preferred that a pure version of the antigen be used in
order to reduce the number of irrelevant antibodies
produced. For soluble antigens, it is preferred to
immunize the animal with a mixture of the antigen and an
20 adjuvant. Adjuvants provide a pool of the emulsified
antigen at the injection site, and this allows the
antigen to be released slowly over an extended period of
time. Immunization may be by the intraperitoneal,
intravenous, subcutaneous or intramuscular routes.
25

Hybridization consists of the preparation of a
myeloma cell line, spleen cells and the process of fusing
these cell lines. The myeloma cell line confers
"immortality" to the antibody; this cell line has the
30 ability to multiply indefinitely and secrete
immunoglobulin. The spleen cells are derived from the
spleen of the immunized animal. The fusion process, in
essence, merges the immortal myeloma cells with the
spleen cells containing antibody to the antigen. The
35 result of the fusion process is typically referred to as
a "hybrid" or, "hybridoma".

The propagation phase, in essence, encourages the growth of hybridoma colonies. Typically, only hybridomas will grow: unfused myeloma cells and unfused spleen cells cannot propagate. Hybridoma colonies are placed into growth-stimulating medium and incubated until sufficient colonies have propagated.

In the screening phase, hybridoma colonies producing antibody against the antigen are identified. Generally, any assay for antibody against the antigen can be used for screening. For example, a labelled anti-immunoglobulin can be used to detect the presence of immunoglobulin.

Cloning ensures that the cells producing antibody comprise a monoclonal population. I.e., the hybridomas selected in the screening phase may comprise antibodies which lack the requisite monoclonality. In essence, cloning involves the setting up of sample-cell cultures, each cell intended to grow into a colony of identical cells. Three different approaches may be used: limiting dilution; semisolid agar culturing; and selective isolation by flow sorting. Limiting dilution is typically preferred. This process comprises plating out in individual culture wells a suspension of hybridoma cells at a dilution such that, statistically, the number of cells in any particular well is from about 0 to about 10 cells. Re-cloning (i.e. repeating the foregoing) is typically recommended. In order to ensure that the clones all produce antibody of the same specificity, the antibody is analyzed by, e.g., electrophoresis or rate nephelometry.

The production of polyclonal antibodies comprises the following steps: Immunization and Purification. Immunization is substantially the same as

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with monoclonal antibody immunization. Purification involves the removal of a sample of the immunized animal's body fluid and purifying it by insolubilizing the antigen on an appropriate affinity gel and pouring
5 the body fluid over such gel; bound antibody can then be removed by, e.g., elution or displacement chromatography. Goats, rabbits or mice can be used as the animal, with rabbits being preferred.

10 Antibodies can also be derived by recombinant DNA technology as outlined above. I.e., the gene or relevant portion of the gene of a cell immunized against an antigen can be removed and inserted into a suitable cloning vector. The recombinant vector can then be
15 transfected into an appropriate host under conditions whereby recombinant antibodies are secreted.

Heretofore, the intentional production of antibodies to SLO (i.e., other than those that are
20 produced by a host in response to infection by SLO-secreting bacteria), has been hampered because of the lethal affects of wild-type SLO on laboratory animals. as used herein, the term "wild-type SLO" is meant to indicate SLO that is naturally secreted by bacterial
25 sources such as, for example, *S. pyogenes*. A need therefore exists for such antibodies, which could be used, for example, for the purification of wild-type, derivative or variant SLO, for immunodiagnostic assays, for therapeutic purposes and for identification purposes.

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SUMMARY OF THE INVENTION

The present invention satisfies the above
needs. Monoclonal, polyclonal and recombinant-derived
35 antibodies can be generated using the SLO derivatives and variants disclosed in the above-referenced and co-pending

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applications. Such antibodies are particularly applicable in at least the following areas: the purification of both wild-type SLO derived from, e.g., bacterial sources, as well as SLO derived by recombinant DNA technology whereby the antibodies are used to bind SLO from the solutions from which the antigenic material was generated; immunodiagnostic assays, including, for example, competitive assays, two-site or "sandwich" assays, and non-competitive assays, whereby the presence of SLO is examined; identification and localization of points of SLO infection whereby labelled antibodies are introduced to a host infected by SLO to determine the point(s) of SLO interaction; and therapeutic areas whereby the antibodies are introduced via a suitable pharmaceutical carrier to a host infected by *S. pyogenes* in an effort to neutralize the resulting secretion of SLO. As further disclosed, an improvement in generating such monoclonal antibodies is provided whereby prior to the immunization step, the rSLO or mSLO is complexed with high density lipoprotein, or introduced to an agent capable of reducing a cysteine amino acid residue.

Ideally, the polyclonal antibodies should have substantially no cross-reactivity to antigenic substances secreted by *S. pyogenes* other than SLO, and most preferably, substantially no cross reactivity to antigenic substances other than rSLO and mSLO. Ideally, the monoclonal antibodies have an affinity for wild-type SLO, mSLO, and rSLO of greater than about 10^{-6} , preferably greater than about 10^{-7} , and more preferably greater than about 10^{-8} .

These and other uses and advantages will become apparent as the disclosure proceeds.

DETAILED DESCRIPTION OF
PREFERRED EMBODIMENTS

Heretofore, the production of antibodies to Streptolysin O for use in purification, diagnostic or therapeutic methodologies has been restricted because SLO is lethal when injected in small doses in the animals from which such antibodies are ordinarily derived (i.e., mice, rabbits, etc.). Thus, attempting to obtain such antibodies using wild-type SLO is not a realistic option.

In the co-pending applications referenced above, soluble SLO derivatives which are hemolytically active, recognized by ASO antibodies against wild-type SLO and which are derived via recombinant DNA techniques (hereinafter referred to as "rSLO"), and soluble SLO variants which are non-hemolytically active, recognized by ASO antibodies against wild-type SLO and which are derived via recombinant DNA techniques (hereinafter "mSLO"), are disclosed. The antibodies disclosed herein are based upon rSLO and mSLO.

As used in this disclosure, rSLO indicates an SLO derivative as defined above and having a percent wild-type SLO specific activity of less than about 75%, more preferably between about 5% and 50%, and most preferably about 9%, based upon a wild-type SLO specific activity of 4×10^5 hemolytic units/mg wild-type SLO. As used in this disclosure, mSLO indicates an SLO variant as defined above and having a percent wild-type SLO specific activity of less than about 1.5%, more preferably less than about 0.5%, and most preferably less than about 0.11%, based upon a wild-type SLO specific activity of 4×10^5 hemolytic units/mg wild-type SLO. These values are relative; thus, if percent wild-type SLO specific activity is based upon a wild-type SLO specific

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activity of 1×10^6 hemolytic units/mg, the above values are decreased by a factor of 2.5 (i.e., 75% becomes 30%; 9% becomes 3.6%; etc.).

5 The preceding is detailed because commercially available wild-type SLO is inherently impure such that an actual "specific activity" of wild-type SLO is elusive. Stated again, the specific activity of wild-type SLO presupposes that the material being analyzed is of
10 sufficient purity to establish an accurate specific activity; however, because commercially available wild-type SLO is inherently impure, a single "correct" specific activity cannot be established.

15 The specific activity of wild-type SLO has been described as being as high as 1×10^6 hemolytic units/mg, although specific activity of 4×10^5 hemolytic units/mg have also been described. Alouf, J. E. "Streptococcal Toxins (Streptolysin O, Streptolysin S, Erythrogenic Toxin)." Pharmac. Ther. II: 661-717 (1980), which is
20 incorporated herein by reference. As noted in our co-pending applications, the specific activity and percent hemolytic activity of specific versions of rSLO and mSLO (rSLO.3 and mSLO.3/6 respectively), based upon the
25 "specific activity" of wild-type SLO, are as follows:

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TABLE I

	Wild-Type SLO	rSLO.3	mSLO.3/6
Specific Activity (Hemolytic Activity in Hemolytic Units/mg)	a) 1×10^6 b) 4×10^5		
		3.6×10^4	1.4×10^4
Percent Hemolytic Activity of Wild-Type SLO	a) 100 b) 100	3.6 9	1.4×10^{-3} 3.5×10^{-3}

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The variant, mSLO.3/6, differs from the derivative, rSLO.3, by a single amino acid. The foregoing indicates that purified rSLO.3 is about 3.6% less hemolytically active than wild-type SLO and that purified mSLO.3/6 is about $1.4 \times 10^{-3}\%$ less hemolytically active than wild-type SLO.

EXAMPLE 1

In Vivo Toxicity Effects of rSLO and mSLO

In order to evaluate in vivo toxicity effects of rSLO.3 and mSLO.3/6, Balb/c mice were administered undiluted and diluted intravenous injections of rSLO.3 and mSLO.3/6. Undiluted and diluted control suspension buffer was administered to an equivalent number of mice. To improve the intravenous injections, the mice were warmed under a heat lamp for 20-30 minutes of pre-injection. Approximately 20 mice were used for each condition.

For the undiluted rSLO.3 and mSLO.3/6, each mouse received an approximate dosage of 17 mg/kg, while for the diluted rSLO.3 and mSLO.3/6, each mouse received an approximate dosage of 1000 μ g/kg. Control solution buffer did not affect the control mice.

Aside from minor ruffling for several minutes after injection, none of the mice receiving either diluted or undiluted rSLO.3 or mSLO.3/6 showed any ill effects from the intravenous administrations.

The foregoing toxicity results indicate that rSLO and mSLO can be utilized in the production of

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monoclonal, polyclonal and recombinant DNA derived antibodies to SLO.

5 In the course of generating monoclonal
antibodies to rSLO and mSLO, Applicants have also
discovered an improvement in the process used for the
production of such antibodies. That improvement relies
upon interaction of the rSLO or mSLO to high density
lipoproteins or in introducing the rSLO or mSLO to an
10 agent capable of reducing a cysteine amino acid residue.
These improvements are set forth in detail in Example 2,
Preparation of Hybridomas.

EXAMPLE 2

15 Preparation of Hybridomas

Hybridomas capable of making monoclonal
antibody with a specific affinity for rSLO and mSLO were
prepared. The materials utilized were as follows. The
20 myeloma cells used were derived from P3X63-AG8.653
myeloma cell line, a non-secreting mouse myeloma line
described by Kearny et al. J. Immunol., 123:1548 (1970).
Myeloma cells can be derived from interspecies hybrids,
such as, for example SMH-D33, a mouse-human
25 "heteromyeloma" fusion partner. Teng et al. Proc. Natl.
Acad. Sci. USA 80:7308 (1983). Alternatively, myeloma
cell lines derived from humans offer the opportunity to
develop human monoclonal antibodies; such would be
preferred for use in, for example, therapeutic
30 situations. Human myeloma cell lines are described in
Nilsson et al J. Clin. Exp. Immunol. 7:477 (1970) ("U-
266") and Matsuoka et al Proc. Soc. Exp. Biol. Med.
125:1246 (1969) ("RPMI-8226"). The foregoing references
are incorporated herein by reference.

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Spleen cells used were obtained from Balb/c mice immunized according to the procedure disclosed below. The growth media was DME low glucose (Irvine Scientific, Santa Ana, CA.), supplemented with 10% fetal calf serum (Gemini Bio, Products, Calabasas, CA) and 2mM L-glutamine (Irvine Scientific). The used media was growth media from three-day culture of 653.1 cells, centrifuged and filtered through a $.22\mu$ filter to remove cells. Conditioned Hypoxanthine Aminopterin Thymidine ("CHAT") media was 50% growth media and 50% used media with 100 units/ml of penicillin-streptomycin solution (Irvine Scientific), 4×10^{-7} M aminopterin (Sigma, St. Louis, M.O.), 10×10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine (both from GIBCO, Grand Island, N.Y.), and 10 units/ml insulin (Eli Lilly, Indianapolis, Ind.). The conditioned media was 50% growth media - 50% used media and 2.5×10^{-5} M b-mercaptoethanol (Sigma). Polyethylene glycol ("PEG") with a molecular weight between about 1300 and 1600 (Sigma) was used. Injection media was DME low glucose with 100 units/ml penicillin-streptomycin solution (both from Irvine Scientific). One-half milliliter of Pristane™ solution (2,6,10,14-tetramethylpentadecane; Aldrich) was injected intraperitoneally into each Balb/c mouse two weeks prior to hybridoma injection.

Hybridomas were constructed in accordance with the method described by Kohler and Milstein, Nature 256:495 (1975). The spleen from the immunized mouse was aseptically removed after cervical dislocation and was ground in a tissue sieve until a single-cell suspension was obtained. After washing, the cells were mixed with the washed 653.1 myeloma cells in a 2:1 ratio of spleen to myeloma cells and then pelleted. The supernatant was removed and the PEG added slowly over one minute. PBS was added to bring the total volume to 22ml and the cells were then pelleted after 8 minutes from the initiation of

PEG addition. The pellet was resuspended in 200 ml of CHAT media and 0.2ml of the suspension was added to each well of ten 96-well (microtiter plates (960 wells total)). The wells were supplied with fresh CHAT on day 6 or 7 post-fusion.

Testing the wells for growth began on day 10 and continued over the next 3-4 days. One-fifth milliliter of rSLO.3 (final concentration: 1.5 μ g/ml coating buffer, 1.59g Na₂CO₃, 2.93g NaHCO₃, 1L triple distilled H₂O, pH 9.6) was dispensed into an appropriate number of microtiter plate wells; these plates, covered and sealed with parafilm, were stored at 5-10°C for at least 3 days prior to use. Hybridoma candidate supernatants were standardized in PBS-0.05% TWEEN 20 (polyoxyethylene sorbitan monolaurate; Sigma), pH 7.2. Antigen-coated microtiter well plates were washed with PBS 0.05% TWEEN 20, followed by aspiration of all liquid from the individual wells. Each well was then filled with 200 μ l of the supernatant from hybridoma candidates, and covered. Plates were then covered and incubated for 2 hrs. in a 37°C incubator, followed by washing (three times) with PBS-0.05% TWEEN 20, and followed by addition of a working dilution of rabbit anti-mouse conjugate (Sigma). Each well was then aspirated to dryness, followed by addition of 200 μ l of the alkaline phosphatase substrate. Alkaline phosphate substrate was prepared as follows. 10mg p-nitrophenyl phosphate (Sigma) was dissolved for each 1 ml of diethanolamine buffer. Diethanolamine buffer was prepared by dissolving .203 g MgCl₂ (Mallinckrodt) in 100 ml double distilled water, followed by the addition of 95.87 ml of diethanolamine (Mallinckrodt) in 750 ml double distilled water; Ph was adjusted to 9.8 with HCl and NaOH. Final volume of one liter was achieved by the addition of double distilled water. Working dilution is defined as 1mg p-nitrophenyl

phosphate per 1ml of diethanolamine buffer and was achieved by diluting the p-nitrophenyl phosphate-diethanolamine buffer in diethanolamine buffer.

5 Plates were then covered and incubated for 30 minutes in a 37°C incubator. Thereafter, 50µl of 2N NaOH was added to each well, followed by gentle shaking, to stop the reaction. Thereafter, optical density readings from microtiter wells were obtained with a Molecular
10 Devices reader set at 405 nm.

 Wells with an optical density reading greater than negative controls were retested the following day. If the reading remained greater than the negative control
15 on the second day of testing, the colony was considered positive and cloned.

 Cloning was carried out by the limiting dilution method, whereby two 96-well plates were
20 utilized, one with 5 cells per well in conditioned media and one with 1 cell per well in conditioned media. One week after cloning, single-colony wells were tested by enzyme immunoassay ("EIA"), as described above. If all wells were positive, the line was considered pure and was
25 recloned a second time for stability. If all the wells did not test 100% positive, a positive well was used for the second cloning. The plates were again tested 7 days after the cloning. This procedure was repeated until all the clones tested 100% positive. The cells were then
30 expanded in growth media and injected in injection media into the peritoneal cavity of Pristane-primed Balb/c mice at a concentration of 3×10^6 hybridoma cells per mouse.

 Prior to injection, supernatant from the
35 cultured cells was used for isotyping by the Ouchterlony gel diffusion method, Acta. Path. Microbiol. Scand.

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26:507 (1949). Ascites fluid was harvested from the mice about 10 days after the mice had been injected with the hybridoma cells. The ascites fluid was then titered by EIA, and the IgG content was measured using an ICS™ rate nephelometer (Beckman Instruments, Inc., Fullerton, CA.).

Immunization protocols were as follows. For the mice designated SLO AS11 and SLO AS12, 20μg of rSLO.3-RA (to be described infra) in Freund's Complete Adjuvant ("FCA") (1.5 ml Aracel A (Mannide Monooleate); 8.5 ml Bayol F (paraffin oil); 5 mg mycobacterium butyricum, killed and dried) was injected intraperitoneally followed at six weeks by intraperitoneal injection of 20μg of rSLO.3-RA in Freund's Incomplete Adjuvant ("FICA"); FICA differs from FCA in that it does not include therein mycobacterium butyricum.. Approximately one month later, 10μg of rSLO.3-RA in FICA was injected intraperitoneally. Three weeks later (three days prior to fusion), 5μg of rSLO.3-RA in PBS was injected intraperitoneally. For mice designated SLO AS13-AS21 (inclusive, without an AS18), 10μg of rSLO.3-HDL (to be described infra) in FCA was injected intraperitoneally, followed at six weeks by intraperitoneal injection of 20μg of rSLO.3-HDL in FICA. Approximately nine weeks later (three days prior to fusion), 5μg of rSLO.3-HDL in PBS was injected intraperitoneally.

Two of the monoclonal antibody candidates were identified as capable of blocking the hemolytic activity of rSLO.3. These two antibodies were derived from SLO AS16 and SLO AS21, respectively. Identification protocol was as follows. To one-half milliliter of PBS (pH 7.0) was added .08μg of rSLO.3 and .2μl of delipidated ascites fluid. Delipidated ascites fluid was prepared by mixing in a 1:1 concentration ratio the ascites fluid from the

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above indicated hybridomas and Beckman Lipid Clearing Solution (Beckman Instruments, Inc.); this mixture was vortexed for at least 1 min. After 5 min. at room temperature, one-half milliliter of rabbit red blood cells (pre-washed once with PBS) was added to the foregoing. After 10 min. at room temperature, the mixture was centrifuged in a Beckman micro-centrifuge at 12,000 rpm for 5 minutes.

A positive result, a result indicating blockage of hemolytic activity by binding of the monoclonal antibody to rSLO.3, was indicated by a clear supernatant and a pellet having a red color (i.e. the pellet comprised red blood cells); a negative result, a result not indicating blockage of hemolytic activity, was indicated by a reddish colored supernatant (i.e. lyses had occurred). The microcapsules containing antibody secreted from the SLO AS16 and SLO AS21 evidenced a clear supernatant, i.e. a positive result.

As noted above, the mice were immunized with either rSLO.3-RA or rSLO.3-HDL. Manipulation of rSLO.3 prior to immunization was conducted based on two properties of wild-type SLO. First, secreted wild-type SLO tends to initially bind to cholesterol within the host. Second, SLO has a single Cys amino acid. These manipulations involved the formation of rSLO-High Density Lipoprotein fractions ("rSLO-HDL") mixtures, and introduction of rSLO to a reducing agent ("rSLO-RA"). These manipulations are also applicable to rSLO, mSLO, and mSLO.3/6.

While not wishing to be bound to any particular theory, Applicants postulated that immunization with rSLO.3-HDL would lead the immune system of the host to recognize the HDL portion of the complex as a foreign

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substance, thus provoking an immune response. Along with the response to the HDL, the immune system of the host would be "sensitized" to the presence of rSLO.3 in the complex and thus antibodies would also be generated by the host relative to the epitopic sites on the rSLO.3. Applicants similarly postulated that the binding of rSLO.3 to HDL leads to a different conformational structure which leads to an immunogenic response to the rSLO.3 epitopic sites. With respect to rSLO-RA, Applicants theorized that the reducing agent interacts with the Cys amino acid of rSLO.3, thus leading to the separation of two rSLO.3 protein structures joined via a single disulfide bond and the formation of -SH groups on each of the two rSLO.3 structures. These rSLO.3 structures would then be capable of forming mixed disulfide bonds between a single rSLO.3 protein structure and material in the adjuvant comprising an -SH group. Such an rSLO.3-adjuvant complex would be similar to the rSLO.3-HDL complex described. I.e., "similar" in the sense that the adjuvant portion of such a complex would be recognized by the host's immune system as a foreign substance, thus leading to sensitization of that system to the rSLO.3 portion of the complex.

High density lipoprotein fractions can be from any mammalian source, and most preferably, the mammalian source is not the same as the mammalian host to be immunized. Accordingly, any HDL is applicable to the present invention, with Bovine HDL Cholesterol and Human HDL Cholesterol being particularly preferred. A most preferred HDL is Pentex® Human Cholesterol Concentrate II (Miles Laboratories, Kankakee, Ill. Source: human plasma, about 24% HDL/cholesterol). The weight ratio of rSLO to HDL (rSLO:HDL) is between about 10:1 to about 1:1, preferably between about 6:1 and about 1:1, and most preferably about 2:1.

Reducing agents which can be utilized include mercaptoethanol, sodium borohydride, sodium cyanoborohydride, sodium bisulfite, sodium thiosulfate, ascorbic acid (vitamin C), uric acid, dithioerythritol, and dithiothreitol. Of these, dithiothreitol is a particularly preferred reducing agent. The concentration of the reducing agent is preferably between about 2.0mM about 8.0mM, more preferably between about 3.0mM and about 7.0mM, and most preferably about 5.0mM.

For rSLO.3-RA, 20 μ g of rSLO.3 was dissolved in 5.0 mM DTT in PBS. This mixture was heated at 37°C for 30 minutes prior to immunization. For rSLO.3-HDL, rSLO.3 (1 mg/ml) was mixed with of Pentex® Human Cholesterol Concentrate II in a 6:1 volume to volume ratio (rSLO.3:Pentex®) prior to immunization.

The hybridomas prepared by this method were capable of producing monoclonal antibody with a specific affinity for rSLO.

EXAMPLE 3

Binding of SLO Monoclonal Antibodies to Wild-Type SLO

While the monoclonal antibodies derived from the SLO AS16 and SLO AS21 hybridomas were able to bind to rSLO.3, it was not an absolute that these would also be capable of binding to wild-type SLO. Stated again, while it is expected that a monoclonal antibody having the requisite monoclonality will have a specific affinity for the antigenic material to which it is specific, there is no guarantee that such a monoclonal antibody will have a specific affinity for any other substance. Accordingly, investigations were conducted to determine if these

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monoclonal antibodies also had a specific affinity for wild-type SLO.

Wild type SLO was obtained from DIFCO Laboratories (Detroit, Michigan, Code 0482). The DIFCO SLO is a desiccated, standardized filtrate of Streptolysin O, in reduced form, prepared from group A streptococcus. The DIFCO SLO was rehydrated in water according to supplier directions just prior to use.

To 0.5 milliliter of rehydrated DIFCO SLO was added 20 μ l of a 1/100 dilution of delipidated ascites fluid that comprised the designated monoclonal antibodies in PBS. The mixture was vortexed for at least about 1 sec. After 5 min. at room temperature, one-half milliliter of rabbit red blood cells (pre-washed once with PBS) was added to the foregoing. After 10 min. at room temperature to mixture was centrifuged in a Beckman micro-centrifuge at 12 rpm for 5 minutes.

A positive result, that is, a result indicating blockage of hemolytic activity by binding of the designated monoclonal antibodies to the wild-type SLO, was indicated by a clear supernatant and a red-colored pellet. A negative result, one not evidencing blockage of hemolytic activity, was indicated by a reddish-colored supernatant. Microfuge tubes containing the designated monoclonal antibodies evidenced a clear supernatant, i.e. a positive result.

The results indicate that the designated monoclonal antibodies derived from the SLO AS16 and SLO AS21 hybridomas and which are specific as to rSLO.3, were capable of sufficiently binding to wild-type SLO to prevent lysis of the red blood cells. Thus, not only do these monoclonal antibodies have a specific affinity for

5 rSLO.3, they additionally have a specific affinity for wild-type SLO. Upon binding thereto, the hemolytic activity of wild-type SLO is substantially prevented by the monoclonal antibodies. Thus, the hybridomas prepared in Example 2 were capable of producing monoclonal antibody with a specific affinity for wild-type SLO.

10 These results indicate that there are a variety of uses for such SLO monoclonal antibodies. For example, such SLO monoclonal antibodies can be used in the purification of wild-type SLO and in assays to determine SLO. It is to be understood that the following are not limited solely to SLO monoclonal antibodies; SLO polyclonal antibodies and recombinant DNA derived SLO
15 antibodies are equally applicable. Most preferably, monoclonal antibodies specific to SLO derivatives or SLO variants are utilized, more preferably monoclonal antibodies specific to rSLO.3, and most preferably, the monoclonal antibodies derived from the SLO AS16 and SLO
20 AS21 hybridomas.

A) Purification of Wild-Type SLO

25 Purification of wild-type SLO can be accomplished using monoclonal antibodies specific to, e.g. SLO derivatives, by coupling such monoclonal antibodies to a suitable solid support.

30 SLO monoclonal IgG, either freshly prepared or previously frozen and thawed, may be utilized. Although the monoclonal antibody may be bound to any material which itself does not have a high affinity for protein, materials such as glass beads, agarose and derivatives thereof are preferred. Most preferred is Bio-Rad Affi-Gel 10.[™] Methods for coupling monoclonal antibodies to
35 such materials are known. For example, 100mg of such

monoclonal antibodies in 30ml of 0.1M MOPS buffer, pH. 7.5 is prepared and ten milliliters of unwashed Bio-Rad Affi-Gel 10 is then added thereto. The resulting slurry is allowed to react at room temperature for 2 hours, followed by centrifugation at 1500 rpm in, for example, a Beckman TJ-6 centrifuge. The supernatant is then removed and the remaining gel-pellet is washed exhaustively with L, e.g., distilled water, and then with PBS.

Five milliliters of the monoclonal antibody bound to affinity gel is then incubated at 4°C for 4 hours with 200ml of fresh or rehydrated wild-type SLO derived from, e.g., *S. pyogenes* culture broths. The resulting slurry consisting of the wild-type SLO bound to monoclonal antibody bound to the affinity gel is loaded onto a small Bio-Rad Econo-Column and washed with 100ml of PBS at 4°C.

Removal of the bound wild-type SLO can be accomplished by methods well known to those in the art, although elution chromatography is preferred. For example, by flushing the column with deionized water, the ionic strength of the column is lowered such that the wild-type SLO is eluted. Similarly, use of buffer having a pH of about 6.0 or less can be used for the elution of the wild-type SLO although a buffer having a pH of greater than about 6.0 may achieve similar results. Elution reagents are well known and can include, for example, high pH reagents (100mm triethylamine, pH 11.5; 100mm phosphate acid, pH 12.5); low pH buffers (100mm glycine, pH 2.5; 100mm glycine, pH 1.8); high salt buffers (5m LiCl, 10mm phosphate, pH 7.2; 3.5-MgCl₂, 10mm phosphate, pH 7.2); ionic detergents (1% SDS; 1% DOC); disassociating agents (2m urea; 8m urea; 2m guanidine HCl); chaotropic agents (3m thiocyanate); and organic

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solvents (10% dioxane; 50% ethylene glycol, pH 11.5; 50% ethylene glycol, pH 8.0).

For ultrapurification, the resulting purified wild-type SLO can then be re-subjected to the same purification protocol.

B) Immunodiagnostic Assays to Determine Levels of Streptolysin O.

10

Examples of immunodiagnostic assays includes nephelometric or turbidimetric assays wherein at least two SLO monoclonal antibody specific for different epitopic regions of SLO are biotinylated. These biotinylated monoclonal antibodies (or fragments thereof) are admixed with a sample suspected of containing SLO, and avidin.

20

Avidin is a relatively large macromolecular protein found in egg whites, and contains four subunits. Biotin is a relatively small, stable, water soluble vitamin. Each of the four avidin subunits of an avidin molecule is capable of specifically binding to a molecule of biotin. Thus, the biotinylated SLO monoclonal antibody conjugates can become bound to a molecule of avidin.

25

In turbidimetry, the reduction of light transmitted through the suspension of particles, or aggregates, is measured. The reduction is caused by reflection, scatter, and absorption of the light by the aggregates. In nephelometry, it is light scattered or reflected toward a detector set not in the direct path of light which is measured. Therefore, if biotinylated SLO monoclonal antibodies, avidin and a sample suspected of containing SLO are admixed, and the mixture is subjected

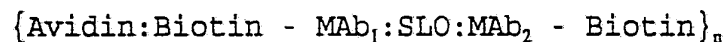
35

to turbidimetric or nephelometric protocols, the amount of SLO present in such sample can be determined.

Turbidimetric and nephelometric protocols are well known and will not be set forth in detail here. See,

5 Sternberg, J. C., "A Rate Nephelometer for Measuring Specific Proteins by Immunoprecipitation for Measuring Specific Proteins by Immunoprecipitation Reactions," Clin. Chem., 23:8, 1456-1464 (1977). A particularly useful nephelometer is the Beckman ICS™ nephelometer
10 (Beckman Instruments, Inc.).

Irrespective of the presence of SLO in the sample, the biotinylated SLO monoclonal antibodies will bind to avidin. If SLO is not present in the sample or
15 is present in a limited titer, the formation of aggregates will be limited. However, if SLO is present in the sample, then the SLO monoclonal antibodies can bind thereto, thus increasing the formation of aggregates as follows:



wherein "MAB₁" and "MAB₂" are SLO monoclonal antibodies specific for different SLO epitopic sites, and "n" indicates that the parenthetical is repetitive. It
25 should be noted that the aforementioned technique may also be performed without the necessity of the use of avidin and biotin.

30 SLO monoclonal antibodies can be coupled to, for example, latex particles for use in latex enhanced nephelometry. Such a protocol is similar to the above described nephelometric assay whereby the SLO monoclonal antibody is coupled to latex particles (instead of
35 biotin). These can then bind to SLO in a sample, and thus increase the number of scatter centers. For

example, latex beads having carboxyl groups on the surface thereof (17-29nm, available from IDC) can be coupled to, e.g., SLO monoclonal antibody. The carboxyl groups can be activated with, e.g., a soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide ("EDC") and stabilized with, e.g., n-hydroxysuccinamide ("NHS"); the concentrations thereof are 10mM and 70mM respectively. Excess untreated EDC and NHS are removed by centrifugation and the "activated" beads are resuspended in an appropriate "attachment" mixture. An appropriate attachment mixture can comprise 5mg/ml of SLO monoclonal antibody in PBS and 1% (v/v) TWEEN-20. After an appropriate attachment period, the SLO monoclonal antibody:latex beads are washed twice with PBS by centrifugation, followed by resuspension in PBS.

In a nephelometric procedure similar to that described above, an inhibition assay to determine levels of anti-Streptolysin O can also be performed. In such a protocol, a fixed nephelometric signal is determined by combining a fixed amount of SLO (preferably rSLO or mSLO) and a fixed amount of an SLO monoclonal antibody. Thereafter, a serum sample suspected of containing ASO would be combined with the fixed amount of SLO such that an immuno-reaction takes place. This is followed by the addition of a fixed amount of SLO monoclonal antibody would be added to the immuno-reactive mixture. An inhibition signal is obtained different from the fixed signal to the degree that ASO is in the sample; i.e., as the amount of ASO in the sample increases, the signal for the immuno-reaction will be different than the fixed signal obtained from the SLO-SLO monoclonal antibody signal.

Alternatively, the Fab' or Fab portions of the SLO monoclonal antibodies can be incorporated, or coated

onto a first region of a so-called "dip-stick"; these are capable of travelling along the regions of the dipstick. SLO derivatives, such as, for example, rSLO.3 can be permanently affixed onto a second region above that of the first region, and labelled antibodies to the SLO monoclonal antibodies can be incorporated, coated, or permanently affixed, onto a third region above the second region. The dipstick, which operate by capillary action, is inserted into a sample suspected of containing SLO such that the sample is able to travel from the first region, through the second region, and then the third region. If SLO is in the sample, it will become bound to the SLO monoclonal antibodies; capillary action will carry both bound and unbound SLO monoclonal antibodies into the second region. Those that are unbound, however, will become bound to the immobilized SLO in the second region; thus, only SLO-SLO monoclonal antibody complexes will be capable of travelling to the third region. In this region, the labelled anti-SLO monoclonal antibodies will bind to those complexes such that, depending upon the label (e.g., radioactive, chemiluminescent, bioluminescent, enzymatic, etc.), the labelled complexes can be either read directly or subjected to further chemical analysis prior to reading the label. Suitable dipsticks which can be utilized in conjunction with such monoclonal antibodies are well-known and will not be discussed herein in detail. See, for example, U.S. Patent No. 5,013,669.

Competitive binding assays are also practical whereby SLO (wild-type, SLO derivatives such as , for example, rSLO.3, or SLO variants, such as, for example, mSLO.3/6) is labelled with, for example, radioactive label, chemiluminescent label, bioluminescent label, fluorophore, enzyme, biotin, etc. using known labelling techniques. Preferably, the SLO derivative is rSLO.3.

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In such an assay, a known amount of the labelled SLO derivative is admixed with insolubilized SLO monoclonal antibody and a sample suspected of containing SLO. Under these parameters, the labelled SLO and sample SLO will compete with binding to the insolubilized SLO monoclonal antibody such that the amount of insolubilized label is inversely proportional to the amount of SLO in the sample.

Two-site called sandwich assays can also be utilized whereby at least one from each of the following reagent Groups A, B and C are admixed: Group A comprises insolubilized SLO polyclonal antibody, SLO antibody produced by recombinant DNA techniques, or SLO monoclonal antibody specific for a first SLO epitopic site; Group B comprising a known quantity of labelled SLO polyclonal antibody, SLO monoclonal antibody specific for a different SLO epitopic site other than the first site, or SLO antibody produced by recombinant DNA techniques; and Group C comprises sample suspected of containing SLO. Insolubilized ternary complexes comprising insolubilized SLO antibody, SLO from the sample, and labelled SLO antibody are then formed such that the amount of insolubilized label is directly proportional to the amount of SLO in the sample.

The foregoing is by no means exhaustive. Rather the described assays for SLO are exemplars of SLO assays utilizing SLO antibodies. Similar assays will be apparent to those skilled in the art.

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EXAMPLE 4

Preparation of Polyclonal
Antibodies To rSLO

5 In order to prepare antibodies to rSLO, 25 μ g of
rSLO.3 was administered intramuscularly to each of two
legs of a rabbit. This was accomplished by mixing equal
volumes of rSLO.3, at an initial concentration of 0.2
mg/dl in phosphate-buffered saline ("PBS"), with FCA.
10 This inoculum was then emulsified until a frothy-texture
was achieved.

 One-half milliliter of the inoculum was
administered as described. One-fifth milliliter of the
15 inoculum was also administered intradermally to each of
ten dorsal sites of the rabbit.

 Approximately one month later, the identical
protocol was followed with one exception. For the
20 secondary immunization, the rSLO.3 was mixed with an
equal volume of 50% FCA and 50% FICA.

 Booster injections of 25 μ g of rSLO.3 were given
monthly for several months. The booster injection
25 protocol is identical to that described above, with two
exceptions. For booster injections, rSLO.3 is combined
in equal volumes of FICA, and one-fifth milliliter of the
inoculum was administered intradermally to fifteen dorsal
sites of the rabbit.

30

EXAMPLE 5

Preparation of Polyclonal
Antibodies to mSLO

35 Polyclonal antibodies to mSLO were also
prepared, following the protocol described in Example 4

with the following exceptions: a) 100 μ g of mSLO.3/6 was administered intramuscularly to each of two legs of two rabbits; and b) the initial concentration of mSLO.3/6 was 7.0 mg/dl.

5

EXAMPLE 6

Purification of rSLO

Polyclonal Antibody

10

The rabbit anti-rSLO.3 polyclonal antibody prepared by the method of Example 4 is purified according to procedures well known in the art. A particularly preferred purification protocol is affinity purification.

15

20

25

SLO (wild-type, an SLO derivative such as, for example, rSLO or an SLO variant, such as, for example, mSLO) bound to affinity gel, is prepared by first solubilizing 100 mg of SLO in 30 ml of 0.1 M MOPS buffer, pH 7.5. Ten milliliters of unwashed Bio-Rad Affi-Gel 10TM affinity gel is then added to the SLO solution. The resulting slurry is allowed to react at room temperature for 2 hours. The reacted slurry is centrifuged at 1500 rpm; a preferred centrifuge is a Beckman TJ-6TM centrifuge. The supernatant is removed and the remaining gel-pellet is washed exhaustively with distilled water and then with PBS.

30

Five milliliters of the SLO bound to the affinity gel is then incubated at 4°C for 4 hours with 200 ml of pooled rabbit anti-rSLO.3 antisera obtained from the method of Example 2. The resulting slurry consisting of the rabbit anti-rSLO.3 antibodies bound to SLO bound to the affinity gel is loaded onto a small Bio-Rad Econo-Column and washed with 100 ml of PBS at 4°C.

35

Methods for releasing bound polyclonal antibody are well known in the art. Particularly preferred is

-30-

elution chromatography, as previously described in Example 3,A.

The foregoing method provides purified polyclonal antibodies to rSLO. Preferably, the polyclonal antibodies to rSLO should have substantially no cross reactivity with S. pyogenes antigens other than SLO, and most preferably, substantially no cross reactivity with antigens other than rSLO.

EXAMPLE 7

Purification of mSLO Polyclonal Antibody

The purification protocol of Example 6 is also applicable to pooled rabbit anti-mSLO.3/6 antisera obtained from the method of Example 5, such that purified polyclonal antibodies to mSLO are provided. Preferably, the polyclonal antibodies to mSLO should have substantially no cross reactivity with S. pyogenes antigens other than SLO, and most preferably, substantially no cross reactivity with antigens other than mSLO.

EXAMPLE 8

SLO Antibodies by Recombinant DNA Techniques

SLO antibodies can be generated using recombinant DNA techniques. Under this approach, wild-type SLO, SLO derivatives or SLO variants (as defined) can be utilized to obtain SLO antibodies using standard immunization protocols such as those described above. Thereafter, the B-cell producing organ (e.g., spleen, PBL or lymph nodes) is removed from the source of immunization. The lymphocytes are then isolated,

followed by isolation of mRNA. cDNA is then synthesized from the isolated mRNA such that cDNA libraries are obtained. Methodologies for cDNA cloning, such as those set forth in Sambrook, J. et al Molecular Cloning: A Laboratory Manual, 2d Edition, Cold Spring Harbor Laboratory Press (1989) Vol. 1-3, and Perbal, Bernard, A Practical Guide to Molecular Cloning, 2d Edition, John Wiley & Sons, New York (1988), which are incorporated herein by reference, can be utilized. Once the cDNA is obtained, an antibody expression library can be generated, using, for example, ImmunoZap™ cloning and expression systems (available from Stratacyte, La Jolla, CA., USA). See also, Sastry, L. "Cloning of the immunological repertoire in Escherichia coli for generation of monoclonal catalytic antibodies: construction of a heavy chain variable region-specific cRNA library," PNAS, USA; 86:5728-5732 (1989), which is incorporated herein by reference.

An alternative procedure can similarly be utilized. Antibodies, including SLO antibody, have an approximate molecular weight of between about 100,000 to 130,000 kD. With an average molecular weight for each amino acid of about 110 kD, a 100,000 kD protein would be encoded by approximately 909 codons, which would be encoded by messages about 2727 bases long; a 130,000 kD protein would be encoded by approximately 1182 codons, which would be encoded by messages about 3545 bases long. Thus, from a conservative perspective, purification of cDNA made from the B-cell producing organ, expressing SLO antibody and which are larger than about 3545 base pairs is preferred; purified fragments greater than about 2727 base pairs can be also be utilized. Thereafter, the isolated cDNA fragments are purified using standard techniques and ligated into an appropriate expression vector (such as, for example, λ gt11) which has

corresponding ends capable of annealing to such fragments. The vector is then transfected into an appropriate cell, such as for example, E. coli, under conditions suitable for growth of an SLO antibody genomic library.

Screening can be accomplished by any method known to those in the art. For example, the antibody proteins can be "lifted" from plaques using protein binding filters (e.g. PVDF membranes, Millipore). The filter is then incubated with labelled SLO, or with SLO followed by washing and incubation with labelled anti-SLO antibody. Identification of the plaques expressing the SLO antibodies having affinity for SLO is possible. This method of screening is preferred in that thousands of plaques can be screened per membrane, such that millions of clones can be screened in a relatively short time period.

EXAMPLE 9

Modification of SLO

Antibodies with Enzymes, Biotin

Fluorochromes, and Drug Delivery Particles

SLO antibodies as described can be labelled with enzymes, biotin and fluorochromes. Such labelled SLO monoclonal antibody can be used for, e.g., diagnostic purposes (such as those disclosed above), screening purposes, or therapeutic purposes.

SLO monoclonal antibodies are preferably used, and most preferably, the IgG isotopes thereof. Prior to such labelling, the SLO antibodies are preferably purified. Purification can be by affinity chromatography with either protein A or SLO as the bonded ligand. Purification by high-performance ion-exchange

chromatography can also be utilized. Crane, L.F.
"Purification of Monoclonal Antibodies by High-
Performance Ion-Exchange Chromatography." Chpt. 9,
Monoclonal Antibody Production Techniques and
5 Applications, L. B. Schook, Ed. Marcel Dekker, Inc. N.Y.,
N.Y. (1987) (hereinafter "Monoclonal Antibody Production
Techniques.") The foregoing chapter is incorporated
herein by reference. Purified IgG isotopes are digested
with pepsin to yield $F(ab')_2$, followed by reduction to
10 yield Fab'.

The Fab' fragments may then be labelled in
accordance with the procedures outlined in e.g.,
Ishikawa, E. et al "Modification of Monoclonal Antibodies
15 with Enzymes, Biotin, and fluorochromes and their
Applications." Chpt. 8, Monoclonal Antibody Production
Techniques. The foregoing chapter is incorporated herein
by reference.

20 Targeted delivery of particles functioning as
reservoir or monolithic devices for important
pharmacologic agents is a primary goal for many
therapeutic treatments. These particles, typically
nanometer to micrometer sized colloidal particles, are
25 typically biodegradable and nontoxic, bioadsorptive,
retained by tissues, and exhibit sustained or controlled-
release of pharmacologic agent(s). A monolithic device is
one in which the agent(s) is dispersed in the particle
matrix, while a reservoir device is one in which the
30 agent(s) is encapsulated by the particle. Natural
polymers used in the preparation of particles include
polysaccharides, e.g., starch and cellulose derivatives,
and proteins, e.g., albumin, collagen and gelatin.
Synthetic biodegradable polymers include polylactide,
35 polyamino acids and copolymers of lactide-co-glycolide,
lactide-co-E-caprolactone, N-(2-hydroxy-propyl)-

methacrylamide, polyortho esters and polyanhydrides. Liposomes and lipoproteins are also available for delivery vehicles. Shaw, J. M. et al. "Drug Delivery Particles and Monoclonal Antibodies," Chpt. 15,
5 Monoclonal Antibody Production Techniques. The foregoing chapter is incorporated herein by reference.

The particles can be coupled directly to the SLO antibody or to F(ab')₂ and Fab' fragments thereof.
10 Such coupling methodologies are described in Shaw, J. M., supra although after such coupling procedures will be readily apparent to the skilled artisan. Purification following such coupling is recommended in order to remove uncoupled SLO antibodies or F(ab')₂ and Fab' fragments.
15 Following purification, pharmaceutical agents can be entrapped in the particles. Additionally radioactive isotopes which are typically used for imaging processes in patients can be entrapped in the particles.

20 The specific pharmaceutical agent(s) utilized depend principally on the target of the monoclonal antibody. With respect to SLO, cardiotoxic effects thereof are well known, as well as the association with, e.g. rheumatic fever. Accordingly, one application of
25 SLO monoclonal antibody coupled to particles comprising radioactive isotopes is the identification of specific compartmental and regional areas where SLO specifically interacts. This would then allow for the delivery of specific agents to such areas. For example, if the
30 cardiotoxic effects of SLO are associated with the direct binding of SLO to myocardial tissue, pharmaceutical agents could be appropriately and readily selected to be delivered to such tissue. Furthermore, while the relationship between SLO and rheumatic fever is well
35 documented, the specific interaction is not well understood. If, however, the inflammation of joints

associated with rheumatic fever is caused by SLO binding to the tissues and muscles of the joints, pharmaceutical agents could be appropriately and readily selected to be delivered to such an area. The selection of such isotopes for the identification of the binding areas of SLO are considered to be within the purview of those in the art. Additionally, once such a localized region is identified, the choice of specified pharmaceutical agents to be delivered to such areas for a specified purpose is also considered to be within the purview of the art.

The Examples herein are not to be construed as limited to specific hybridoma cell lines which are preferred. The methodologies described for generating hybridoma cell lines capable of producing monoclonal antibody with an affinity to rSLO, mSLO and/or wild-type SLO are not to be construed as limited solely to preferred hybridoma cell lines. Similarly, the preferred hybridoma cell lines disclosed above in no way constitute an admission, either actual or implied, that these are the only hybridoma cell lines to which Applicants are entitled. They are entitled to the full breadth of protection under applicable patent laws. Additionally, the monoclonal antibodies generated from such cell lines are similarly not limited. Preferred hybridoma cell lines have been identified by Applicants as SLO AS11, SLO AS12, SLO AS16 and SLO AS21. For purposes of claiming the particularly cell lines by designation, the hybridoma cell lines were deposited on July 16, 1991 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. The hybridoma cultures were tested by the ATCC on July 19, 1991, and determined to all be viable cultures. The ATCC has assigned the four

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hybridoma cell lines ATCC deposit numbers HB 10826, HB 10827, HB 10828 and HB 10829, respectively.

Although the present invention has been
5 described in considerable detail with regard to certain
preferred embodiments thereof, other embodiments within
the scope of the teachings of the present invention are
possible. For example, although certain of the disclosed
antibodies are based upon the SLO derivative rSLO.3 and
10 the SLO variant mSLO.3/6, the invention is not limited
thereby. As such, while the production of SLO antibodies
to these specific SLO derivative and variant have been
described in detail, these are to be construed as
exemplars such that other rSLO or mSLO antigenic
15 candidates can be used to generate monoclonal, polyclonal
and recombinant DNA derived antibodies within the spirit
and scope of the disclosure. Accordingly, neither the
disclosure, nor the claims to follow, are intended, nor
should be construed to be, limited by the descriptions of
20 the preferred embodiments contained herein.

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What is claimed is:

1. A monoclonal antibody with an affinity for
rSLO of at least 10^{-6} .
5
2. A monoclonal antibody with an affinity for
mSLO of at least 10^{-6} .
3. A monoclonal antibody with an affinity for
rSLO and wild-type SLO of at least 10^{-6} :
10
4. A polyclonal antibody with an affinity for
rSLO.
5. A polyclonal antibody with an affinity for
mSLO.
15
6. A polynucleotide sequence encoding an
antibody to rSLO.
20
7. A polynucleotide sequence encoding an
antibody to mSLO.
8. A hybridoma cell line capable of secreting
antibody for rSLO.
25
9. The hybridoma cell line of claim 8 wherein
the hybridoma is a hybrid of a B-cell source from an
animal immunized with an adjuvant comprising rSLO and an
animal myeloma cell.
30
10. The hybridoma cell line of claim 9 wherein
the B-cell source is an animal spleen cell.
11. The hybridoma cell line of claim 10
wherein the animal is a mouse.
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12. The hybridoma cell line of claim 9 wherein the myeloma cell is derived from an animal selected from the group consisting of mouse and human.

5 13. The hybridoma cell line of claim 9 wherein the B-cell source animal is a mouse and the myeloma cell source animal is a mouse.

10 14. A hybridoma cell line designated by the ATCC identifier HB 10826.

15 15. A hybridoma cell line designated by the ATCC identifier HB 10827.

16 16. A hybridoma cell line designated by the ATCC identifier HB 10828.

20 17. A hybridoma cell line designated by the ATCC identifier HB 10829.

25 18. An antibody secreted by a hybridoma cell lines selected from the group consisting of hybridoma cell line designated by the ATCC identifiers HB 10826, HB 10827, HB 10828 and HB 10829.

19. A hybridoma cell line capable of secreting antibody to wild type SLO.

30 20. The hybridoma cell line of claim 19 wherein the hybridoma is a hybrid of a B-cell source from an animal immunized with an adjuvant comprising rSLO and an animal myeloma cell.

35 21. The hybridoma cell line of claim 20 wherein the B-cell source is an animal spleen cell.

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22. The hybridoma cell line of claim 21 wherein the animal is a mouse.

5 23. The hybridoma cell line of claim 19 wherein the myeloma cell is derived from an animal selected from the group consisting of mouse and human.

10 24. The hybridoma cell line of claim 19 wherein the B-cell source animal is a mouse and the myeloma cell source animal is a mouse.

25. A hybridoma cell line capable of secreting antibody for mSLO.

15 26. The hybridoma of claim 25 wherein the hybridoma is a hybrid of a B-cell source from an animal immunized with an adjuvant comprising rSLO and an animal myeloma cell.

20 27. The hybridoma of claim 26 wherein the B-cell source is an animal spleen cell.

25 28. The hybridoma of claim 27 wherein the animal is a mouse.

29. The hybridoma of claim 26 wherein the myeloma cell is derived from an animal selected from the group consisting of mouse and human.

30 30. The hybridoma of claim 25 wherein the B-cell source animal is a mouse and the myeloma cell source animal is a mouse.

35 31. An antibody to rSLO.

32. The antibody of claim 31 wherein the antibody has an IgG isotype.

33. An antibody to mSLO.

5

34. The antibody of claim 33 wherein the antibody has an IgG isotype.

35. A process for purifying wild-type SLO comprising the following steps:

10

a) obtaining a source comprising wild-type SLO;

15

b) introducing said source to an insolubilized antibody to rSLO having a specific affinity for wild-type SLO such that said wild-type SLO becomes bound to said insolubilized antibody;

20

c) removing undesired materials from said insolubilized antibody to rSLO; and

25

d) displacing said wild-type SLO by elution thereof wherein the eluate contains purified wild-type SLO.

36. The process of claim 35 wherein the process is repeated at least once.

30

37. The process of claim 35 wherein an elutinator capable of displacing said wild type SLO is selected from the group consisting of deionized aqueous solutions, solutions having a pH less than the pH of said source comprising wild-type SLO, organic solvents, chaotropic agents, dissociating agents, ionic detergents and buffers comprising high salt concentration.

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38. A process for purifying wild-type SLO comprising the following steps:

5 a) obtaining a source comprising wild-type SLO;

10 b) introducing said source to an insolubilized antibody to mSLO having a specific affinity for wild-type SLO such that said wild-type SLO becomes bound to said insolubilized antibody;

c) removing undesired materials from said insolubilized antibody to mSLO; and

15 d) displacing said wild-type SLO by elution thereof wherein the eluate contains purified wild-type SLO.

20 39. The process of claim 38 wherein the process is repeated at least once.

25 40. The process of claim 39 wherein an elutinator capable of displacing said wild type SLO is selected from the group consisting of a deionized aqueous solution solutions having a pH less than the pH of said source comprising wild-type SLO, organic solvents, chaotropic agents, dissociating agents, ionic detergents and buffers comprising a high salt concentration.

30 41. The IgG isotype of claim 27 wherein said isotype is manipulated to yield Fab' fragment portions thereof.

35 42. The Fab' fragment of claim 41 comprising an agent conjugated thereto.

43. The Fab' fragment of claim 34 wherein the agent is selected from the group consisting of enzymes, biotin, fluorochromes, latex, particles, radioactive isotopes, chemiluminescent labels, bioluminescent labels and particles capable of incorporating pharmaceutical agents therein.

44. The monoclonal antibody of claims 1, 2, and 3 comprising an agent conjugated thereto.

45. The monoclonal antibody of claim 44 wherein said agent is selected from the group consisting of enzymes, biotin, fluorochromes, latex particles, radioactive isotopes, chemiluminescent labels, bioluminescent labels and particles capable of incorporating pharmaceutical agents therein.

46. In a process for producing monoclonal antibody having a specific affinity for rSLO, the improvement comprising complexing rSLO to high density lipoprotein and immunizing an animal with the rSLO-high density lipoprotein complex.

47. In a process for producing monoclonal antibody having a specific affinity for mSLO, the improvement comprising complexing mSLO to high density lipoprotein and immunizing an animal with the mSLO-high density lipoprotein complex.

48. In a process for producing monoclonal antibody having a specific binding affinity for rSLO, the improvement comprising immunizing an animal with an adjuvant comprising rSLO and an agent capable of reducing a cysteine amino acid residue.

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49. In a process for producing monoclonal antibody having a specific binding affinity for mSLO, the improvement comprising immunizing an animal with an adjuvant comprising mSLO and an agent capable of reducing a cysteine amino acid residue.

50. The process of claim 48 wherein said reducing agent is selected from the group consisting of mercaptoethanol, sodium borohydride, sodium cyanoborohydride, sodium bisulfite, sodium thiosulfate, ascorbic acid, uric acid, dithioerythritol and dithiothreitol.

51. The process of claim 48 wherein said reducing agent is dithiothreitol.

52. The process of claim 48 wherein the concentration of said reducing agent in said adjuvant is between about 2.0mM and about 8.0mM.

53. The process of claim 48 wherein the concentration of said reducing agent in said adjuvant is between about 3.0mM and about 7.0mM.

54. The process of claim 48 wherein concentration of said reducing agent in said adjuvant is about 5.0mM.

55. The process of claim 49 wherein said reducing agent is selected from the group consisting of mercaptoethanol, sodium borohydride, sodium cyanoborohydride, sodium bisulfite, sodium thiosulfate, ascorbic acid, uric acid, dithioerythritol and dithiothreitol.

56. The process of claim 49 wherein said reducing agent is dithiothreitol.

5 57. The process of claim 49 wherein the concentration of said reducing agent in said adjuvant is between about 2.0mM and about 8.0mM.

10 58. The process of claim 49 wherein the concentration of said reducing agent in said adjuvant is between about 3.0mM and about 7.0mM.

15 59. The process of claim 49 wherein concentration of said reducing agent in said adjuvant is about 5.0mM.

60. The process of claim 46 wherein the high density lipoprotein fractions is from a mammalian source selected from the group consisting of bovine and human.

20 61. The process of claim 46 wherein the weight ratio of rSLO to high density lipoprotein fraction is between about 10:1 and about 1:1.

25 62. The process of claim 46 wherein the weight ratio of rSLO to high density lipoprotein fraction is between about 6:1 and about 1:1.

30 63. The process of claim 46 wherein the weight ratio of rSLO to high density lipoprotein fraction is about 2:1.

35 64. The process of claim 47 wherein the high density lipoprotein fraction is from a mammalian source selected from the group consisting of bovine and human.

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65. The process of claim 47 wherein the weight ratio of rSLO to high density lipoprotein is between about 10:1 and about 1:1.

5 66. The process of claim 47 wherein the weight ratio of rSLO to high density lipoprotein is between about 6:1 and about 1:1.

10 67. The process of claim 47 wherein the weight ratio of rSLO to high density lipoprotein is about 2:1.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/05122

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C12N15/13; C12P21/08; C07K15/28; C12N5/12 C07K3/18; A61K39/385		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C12P ; C07K ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	DERWENT JAPANESE PATENTS REPORT Derwent Publications Ltd., London, GB; AN 90-011073 [02] & JP,A,1 290 698 (FUJI REBIO KK) 22 November 1989 see abstract ---	1-45
X	EP,A,0 369 825 (M. KEHOE) 23 May 1990 see the whole document ---	1-5, 18, 31-34
A	GB,A,2 233 977 (M. KEHOE ET AL.) 23 January 1991 see the whole document ---	1-5, 18, 31-34, 48-59
¹⁰ Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 18 SEPTEMBER 1992	Date of Mailing of this International Search Report 12. 10. 92	
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer NOOIJ F.J.M.	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9205122
SA 61837

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0369825	23-05-90	GB-A, B 2226563 JP-A- 2242683	04-07-90 27-09-90
GB-A-2233977	23-01-91	None	